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In CD28-costimulated human naïve CD4$^+$ T cells, I-κB kinase controls the expression of cell cycle regulatory proteins via interleukin-2-independent mechanisms

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Summary

Stimulation of naïve CD4$^+$ T cells through engagement of the T-cell receptor (TCR) and the CD28 co-receptor initiates cell proliferation which critically depends on interleukin (IL)-2 secretion and subsequent autocrine signalling via the IL-2 receptor. However, several studies indicate that in CD28-costimulated T cells additional IL-2-independent signals are also required for cell proliferation. In this study, using a neutralizing anti-human IL-2 antibody and two selective, structurally unrelated, cell-permeable I-κB kinase (IKK) inhibitors, BMS-345541 and PS-1145, we show that in human naïve CD4$^+$ T cells stimulated through a short engagement of the TCR and the CD28 co-receptor, IKK controls the expression of the cell cycle regulatory proteins cyclin D3, cyclin E and cyclin-dependent kinase 2 (CDK2) and the stability of the F-box protein S-phase kinase-associated protein 2 (SKP2) and its co-factor CDC28 protein kinase regulatory subunit 1B (CKS1B), through IL-2-independent mechanisms.

Keywords: CD4$^+$ T cells; cell cycle; I-κB kinase; I-κB kinase inhibitors; CD28; interleukin-2

Introduction

The transition of eukaryotic cells from G0 to G1 phase, and progression into S phase, are promoted by the sequential activation of complexes of cyclin D and cyclin-dependent kinase 4 (CDK4) or CDK6, cyclin E and CDK2, and cyclin A and CDK2.[1] These proteins are absent or expressed at very low levels in resting T cells, but their expression is rapidly induced following T-cell receptor (TCR)/CD28 costimulation.[2,3] A major consequence of increased cyclin D–CDK4/6 complex levels during G1 phase is the sequestration of the CDK inhibitor p27^{KIP1}. This event releases cyclin E/CDK2 from p27^{KIP1}, facilitating cyclin E/CDK2 activation.[4] Following sequestration, p27^{KIP1} is phosphorylated by cyclin E/CDK2 on Thr 187[5], polyubiquitinated by the RING-

Abbreviations: BrdU, bromodeoxyuridine; BSA, bovine serum albumin; CDK, cyclin-dependent kinase; CKS1B, CDC28 protein kinase regulatory subunit 1B; DMSO, dimethylsulphoxide; EGR-2, early growth response gene 2; ERK-8, extracellular-signal-regulated kinase 8; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; IKK, I-κB kinase; IL-2, interleukin-2; IL-2RA, interleukin-2 receptor, alpha chain; I-κB, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor; KIP, kinase inhibitory protein; NEMO, nuclear factor-κB essential modulator; NFAT, nuclear factor of activated T cell; NF-κB, nuclear factor κB; nIL-2, neutralizing anti-human interleukin-2 monoclonal antibody; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; PVDF, polyvinylidene fluoride; rhIL-2, recombinant human interleukin-2; RPL13A, ribosomal protein L13A; SCF, Rbx1-Skp1–Cul1–F-box-protein ubiquitin ligase; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SKP2, S-phase kinase-associated protein 2; TBP, TATA binding protein; TBS, Tris-buffered saline; TCR, T-cell receptor; β-TRCP, β-transducin repeat-containing protein.

finger-type ubiquitin ligase complex $SCF^{SKP2\text{-}CKS1B}$ (Rbx1-Skp1-Cul1-F box protein; the superscript indicates the F-box protein and ist cofactor)[6–9] and finally degraded by the 26S proteasome[10].

CD28 costimulation of T cells is mirrored by the activation of the canonical nuclear factor (NF)-κB signalling pathway, which is responsible for connecting TCR-proximal signals to the activation of the NF-κB family of transcription factors.[11–14] This pathway centres on the activation of the trimeric I-κB kinase (IKK) complex which has two major catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), plus the regulatory subunit IKKγ/NF-κB essential modulator (NEMO). Activated IKK phosphorylates I-κB proteins on two conserved serine residues, resulting in polyubiquitination by the $SCF^{\beta\text{-}TRCP}$ (β-transducin repeat-containing protein) E3-ubiquitin ligase complex, and degradation by the 26S proteasome. This unmasks the NF-κB nuclear translocation sequence, allowing NF-κB dimers to translocate into the nucleus, where they regulate the expression of genes required for T-cell expansion. Of the two IKK catalytic subunits, IKKβ is responsible for most of the I-κB kinase activity.[15] The recent identification of NF-κB- and I-κB-independent targets of IKK reveals that the impact of IKK activation could be more widespread than previously realized,[15,16] further expanding the potential effects of IKK targeting on cell signalling.

In activated T cells, NF-κB transcription factors, by co-operating with a number of transcriptional regulators, enhance the expression of several genes, including those for the mitogenic cytokine interleukin (IL)-2 and its high-affinity receptor IL-2RA.[17,18] Upon interacting with its receptor, IL-2 elicits the co-ordinated activation of several intracellular signalling pathways that promote entry of T cells into the cell cycle, and clonal expansion. For this reason, CD28 costimulation was proposed to trigger T-cell proliferation through accumulation of IL-2, and subsequent activation of its signalling pathway.[19]

However, a number of observations in CD28-,[20] IL-2-[21] and cytotoxic T-lymphocyte antigen 4 (CTLA4)-deficient[22] mice, as well as in human primary T cells,[3] suggest that in CD28-costimulated T cells additional IL-2-independent cell cycle regulatory mechanisms are required for cell proliferation.

Recent studies have shown that the duration of the TCR/CD28 engagement appears to be a critical factor determining the IL-2 requirement for T-cell proliferation: while a short (20–24 hr) engagement of the TCR and CD28 programmes T cells to proliferate in response to autocrine IL-2, a prolonged (72–96 hr) TCR/CD28 engagement circumvents the need for autocrine IL-2 and supports IL-2-independent lymphocyte proliferation.[3,23,24]

In this study we aimed to determine if, in human naïve $CD4^+$ T cells, stimulated through a short engagement of the TCR and the CD28 co-receptor, signals from IKK promote T-cell proliferation through IL-2-independent cell-cycle regulatory mechanisms. The effects of a neutralizing anti-human IL-2 antibody on the expression of cell-cycle regulatory proteins involved in the G0/G1 transition and S phase entry of CD28-costimulated human naïve $CD4^+$ T cells were compared with the effects of two selective, structurally unrelated, cell-permeable IKK inhibitors, BMS-345541[25] and PS-1145.[26] Our results demonstrate that, in addition to having a pivotal role in the up-regulation of IL-2 and IL-2RA gene expression, proliferative signals from IKK control the expression of the cell-cycle regulatory proteins cyclin D3, cyclin E and CDK2, and the stability of the F-box protein SKP2 and its co-factor CKS1B, through mechanisms independent of IL-2.

Materials and methods

Reagents

BMS-345541[4(2′-aminoethyl)amino-1,8-dimethylimidazol [1,2-a]quinoxaline] (B9935) and PS-1145[N-(6-chloro-9H-pyrido[3,4-b]indol-8-yl)-3-pyridinecarboxamide] (P6624), protease inhibitor cocktail (P8340), antibiotic-antimycotic solution (A5955), Laemmli 2× sample buffer (S3401), phosphate-buffered saline (PBS) (P5493), and β-actin monoclonal antibody (A-5441) were from Sigma-Aldrich (Milan, Italy). Polyclonal antibodies to $p27^{KIP1}$ (sc-528), NF-κB p50 (sc-7178), β-tubulin (sc-9104), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-25778), cyclin D2 (sc-181), cyclin D3 (sc-182), CDK4 (sc-260), CDK6 (sc-177), cyclin E (sc-481) and early growth response gene 2 (EGR-2) (sc-20690), monoclonal antibodies to p65-RelA (sc-71765), CDK2 (sc-6248) and cyclin A (sc-56300), and secondary antibodies conjugated to horseradish peroxidase (HRP) were from Santa Cruz (Heidelberg, Germany). Monoclonal antibodies to lamin-B1 (33-2000) and SKP2 (32-3300), and polyclonal antibody to CKS1B (36-6800) were from Invitrogen (Milan, Italy). Recombinant human IL-2 (11011456001) was from Roche (Milan, Italy).

Polyclonal antibodies to c-ABL (2862) and histone H4 (2592) were from Cell Signaling (Milan, Italy). Monoclonal antibodies to I-κBα (ALX-804-209) and proteasome subunit alpha type 5 (PW-8125) were from Vinci-Biochem (Florence, Italy). Lymphoprep (1114545) was from Sentinel (Milan, Italy). BioWhittaker X-VIVO 15 medium (BE04-418F) was from Lonza (Milan, Italy). Enhanced chemiluminescence (ECL) reagent (WBKL-S0500) and polyvinylidene fluoride (PVDF) (immobilon-P, IPVH00010) were from Millipore Corporation (Milan, Italy). Nitrocellulose (RPN303D) was from Amersham Bioscience (Milan, Italy). Protein molecular markers (SM0671) were from Fermentas (Milan, Italy). Superscript III reverse transcriptase (18080-044), $oligo(dT)_{20}$ (18418-020) and SybrGreen qPCR Super Mix (11733-046) were from Invitrogen. The DC Protein Assay kit (500-

0119) was from Bio-Rad (Milan, Italy). All other chemicals were high grade from Sigma-Aldrich.

Isolation of human naïve CD4+ CD25− T cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Isopaque (Lymphoprep) density gradient centrifugation of buffy coat leukopheresis residues from fresh blood samples from healthy donors. To eliminate potential suppressive effects of $CD4^+$ $CD25^+$ cells on proliferation,[27] $CD4^+$ T cells depleted of $CD25^+$ cells were used throughout the study. $CD4^+$ $CD25^-$ T cells were isolated from PBMCs by negative selection using the Human $CD4^+$ $CD25^+$ Regulatory T Cell Isolation kit (130-091-301) according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Isolated T cells were > 99% $CD4^+$ $CD25^-$, as assessed by flow cytometry analysis.

T-cell stimulation

$CD4^+$ $CD25^-$ T cells (3×10^6) were maintained at 37° in a 5% CO_2 humidified atmosphere in 24-well plates at 2×10^6/ml/cm^2 in X-VIVO 15 medium supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml amphotericin B. Cells were stimulated with $1{\cdot}5 \times 10^6$ MACSiBeads™ particles loaded with anti-CD3, plus anti-CD28 monoclonal antibodies (CD3/CD28 costimulation) according to the manufacturer's instructions (T Cell Activation/Expansion kit; Miltenyi 130-091-441) for the indicated times (see results). Cell viability was evaluated by trypan blue exclusion.

Inhibition experiments

$CD4^+$ $CD25^-$ T cells (3×10^6) were preincubated for 60 min with BMS-345541 or PS-1145 at 0·5–6 μM or drug vehicle [dimethylsulphoxide (DMSO)] and activated as described above. In some experiments, the drugs were replaced by neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2) at 0·02–4 μg/ml (MAB202; R&D Systems, MN).

T-cell proliferation assay

Cell proliferation was evaluated using the BrdU Cell Proliferation Assay kit (HTS01; Calbiochem, Darmstadt, Germany), according to the manufacturer's instructions. Briefly, $CD4^+$ $CD25^-$ T cells (10^4 cells in 100 μl of medium) were seeded into a 96-well culture plate, preincubated for 60 min with nIL-2, BMS-345541, PS-1145 or vehicles, added with 20 μl of BrdU label (1 : 2000) in fresh medium, activated by the addition of MACS iBeads particles loaded with anti-CD3 plus anti-CD28 monoclonal antibodies, and maintained at 37° in a 5% CO_2 humidified atmosphere for the indicated times (see results). In controls, BrdU label was omitted. After incubation, cells were treated with fixative/denaturing solution and incubated with anti-BrdU monoclonal antibody. Unbound antibody was removed by washing and goat anti-mouse HRP-conjugate was added. Following extensive washing, fluorogenic substrate was added and fluorescent product intensity measured at 355 nm (excitation) and 444 nm (emission) using a Fluoroskan Ascent-Thermo microplate fluorometer (Thermo Fisher Scientific, MA). Data are the ratio of the signals obtained from the labelled (BrdU) sample to those obtained from the unlabelled sample (no BrdU) after subtraction of endogenous fluorescence.

Flow cytometry

For CD4 and CD25 expression analysis, cells were washed with PBS supplemented with 0·5% bovine serum albumin (BSA) (A3156; Sigma-Aldrich) and stained for 20 min at 4° with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD25 (Becton-Dickinson, NJ) and Cy-5-conjugated anti-CD3 (Caltag Laboratories, Burlingame, CA) with appropriate isotype control. Cells were washed, resuspended in PBS/BSA and analysed using an EPICS XL Beckman-Coulter, CA flow cytometer. Analysis of DNA content was carried out using propidium iodide staining. Briefly, naïve $CD4^+$ $CD25^-$ T cells (1×10^6) were pretreated for 1 hr with DMSO, 3 μM BMS-345541 or 3 μM PS-1145 and then stimulated for 24 hr with anti-CD3 plus anti-CD28 antibodies. After treatment, cells were washed in PBS and fixed on ice with 70% volume/volume (v/v) cold ethanol to a final concentration of 65% v/v. Fixed cells were washed in PBS, resuspended in propidium iodide (PI) solution (20 μg/ml PBS) containing DNase free RNase A (50 μg/ml PBS), incubated for 30 min at room temperature in the dark and analysed by flow cytometry.[28]

Whole-cell extracts

Cultured cells (3×10^6) were washed with PBS at 4° and extracted on ice in 50 μl of RIPA buffer [50 mM Tris-HCl, pH 7·4, 150 mM NaCl, 1% v/v Triton X-100, 0·25% weight/volume (w/v) sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM NaF, 1 mM Na_3VO_4 and 1 mM $Na_4P_2O_7$] containing 1% v/v protease inhibitor cocktail. Lysate was centrifuged at 18 000 ***g*** for 5 min at 4°, and the supernatant was collected and stored at −80°. Protein concentration was determined using the DC Protein Assay kit.

Nuclear extracts

Cultured cells (3×10^6) were washed with PBS at 4° and nuclear extracts prepared using the ProteoJet Cytoplasmic

and Nuclear Protein Extraction kit (K0311; Fermentas) according to the manufacturer's instructions, with 1% v/v protease inhibitor cocktail. Nuclear and cytosolic extracts were stored at −80°. Protein concentration was determined as above.

Immunoblotting

Whole-cell or nuclear extracts were mixed 1 : 1 with Laemmli sample buffer and heated at 95° for 5 min. Proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using Tris/Glycine[29] or Tris/Tricine[30] buffer systems. Resolved proteins were electro-transferred to PVDF or nitrocellulose membranes, blocked with 5% BSA (RPN412; Amersham) in TBS (20 mM Tris, pH 7·6, and 140 mM NaCl) containing 0·02% v/v Tween 20 (blocking solution) and probed with antibodies as indicated (see results). Immunoreactive bands were detected by ECL using a G:Box Chemi-XT CCD gel imaging system and GeneSnap image acquisition software (Syngene, Cambridge, UK). Relative band intensities were quantitated using GeneTools image analysis software (Syngene).

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from 3×10^6 cells using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Purified RNA was quantified spectrophotometrically, aliquoted and stored at −80°. RNA (1 μg) was converted to cDNA using Superscript III reverse transcriptase and 2·5 μM oligo$(dT)_{20}$ primer in 20 μl, according to the manufacturer's specifications. Real-time PCR was performed on a Bio-Rad Mini-Opticon thermal cycler using 15 ng of reverse-transcribed RNA and 200 nM specific forward and reverse primers in 25 μl, using SybrGreen qPCR Super Mix. PCR conditions were 3 min at 95°, with 50 cycles of 15 seconds at 95° and 30 seconds at 60°. All samples were assayed in triplicate. mRNA levels were normalized using TATA binding protein (TBP) and ribosomal protein L13A (RPL13A) as internal controls[31] using genex software (Bio-Rad). Melting point analysis was carried out for all runs. To measure PCR efficiency, serially diluted, reverse-transcribed mRNA (from 0·1 pg to 200 ng) was amplified with each set of primers, and linear standard curves obtained by plotting the log of the serial dilutions against the cycle threshold (CT) value. The slope of each curve was used to calculate efficiency for primer sets using the formula $E = 10^{-1/\text{slope}}$. The relative expression of the tested genes in untreated and treated cells was determined using the $2^{-\Delta\Delta CT}$ formula.[32] Amplification products for all tested genes were analysed on ethidium bromide-stained agarose gels to ensure single amplification products of the expected size. Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and synthesized by MWG (Martinsried, Germany). IL-2 mRNA (NM_000586) was amplified from position 38 to 264, with primers: forward 5′-acctcaactcctgccacaat-3′ and reverse 5′-gccttcttgggcatgtaaaa-3′. IL-2RA mRNA (NM_000417) was amplified from 892 to 1072, with primers: forward 5′-ggctgtgttttcctgctgat-3′ and reverse 5′-gcgaccatttagcacctttg-3′. CDK4 mRNA (NM_000075) was amplified from 1187 to 1367, with primers: forward 5′-ctggacactgagagggcaat-3′ and reverse 5′-gaaagggacaagagggaaca-3′. CDK6 mRNA (NM_001259) was amplified from 10 933 to 11 119, with primers: forward 5′-ctttcccaagaggcagatga-3′ and reverse 5′-gggtcacaaagcatccctta-3′. CDK2 mRNA (NM_001798) was amplified from 1903 to 2027, with primers: forward 5′-cctgatcccattttcctctg-3′ and reverse 5′-ttttacccatgccctcactc-3′. Cyclin D2 mRNA (NM_001759) was amplified from 3617 to 3831, with primers: forward 5′-gtttttcccctccgtctttc-3′ and reverse 5′-ttgaaaacccgaccgtttag-3′. Cyclin D3 mRNA (NM_001760) was amplified from 615 to 774, with primers: forward 5′-ggacctggctgctgtgattg-3′ and reverse 5′-gatcatggatggcgggtaca-3′. Cyclin E1 mRNA (NM_001238) was amplified from 1625 to 1777, with primers: forward 5′-tacaccagccacctccagac-3′ and reverse 5′-tacaacggagcccagaacac-3′. Cyclin A2 mRNA (NM_001237) was amplified from 1366 to 1587, with primers: forward 5′-ttattgctggagctgcctttt-3′ and reverse 5′-ctggtgggttgaggagagaa-3′. SKP2 mRNA (NM_005983) was amplified from 711 to 924, with primers: forward 5′-catttcagccctttcgtgt-3′ and reverse 5′-gggcaaattcagagaatcca-3′. CKS1B mRNA (NM_001826) was amplified from 532 to 723, with primers: forward 5′-ccagatgagtgctctgtgga-3′ and reverse 5′-ccgcaagtcaccacacatac-3′. TBP mRNA (NM_003194) was amplified from 29 to 219, with primers: forward 5′-cggctgtttaacttcgcttc-3′ and reverse 5′-ttcttggcaaaccagaaacc-3′. RPL13A mRNA (NM_012423) was amplified from 540 to 768, with primers: forward 5′-agctcatgaggctacggaaa-3′ and reverse 5′-cttgctcccagcttcctatg-3′.

Statistical analysis

Data are the mean ± standard deviation (SD) of three independent experiments. Statistical significance was determined using Student's *t*-test. *P*-values < 0·05 were considered statistically significant.

Results

Proliferation of CD28-costimulated T cells is prevented by nIL-2 and IKK inhibitors

Engagement of the TCR together with the costimulatory receptor CD28 programmes naïve T cells to proliferate in response to autocrine IL-2.[23] When purified $CD4^+$ $CD25^-$ human naïve T cells (Fig. 1a) were stimulated with anti-CD3 plus anti-CD28 antibodies, a time-dependent induction of DNA synthesis was observed, which was inhibited in a concentration-dependent manner by nIL-2.

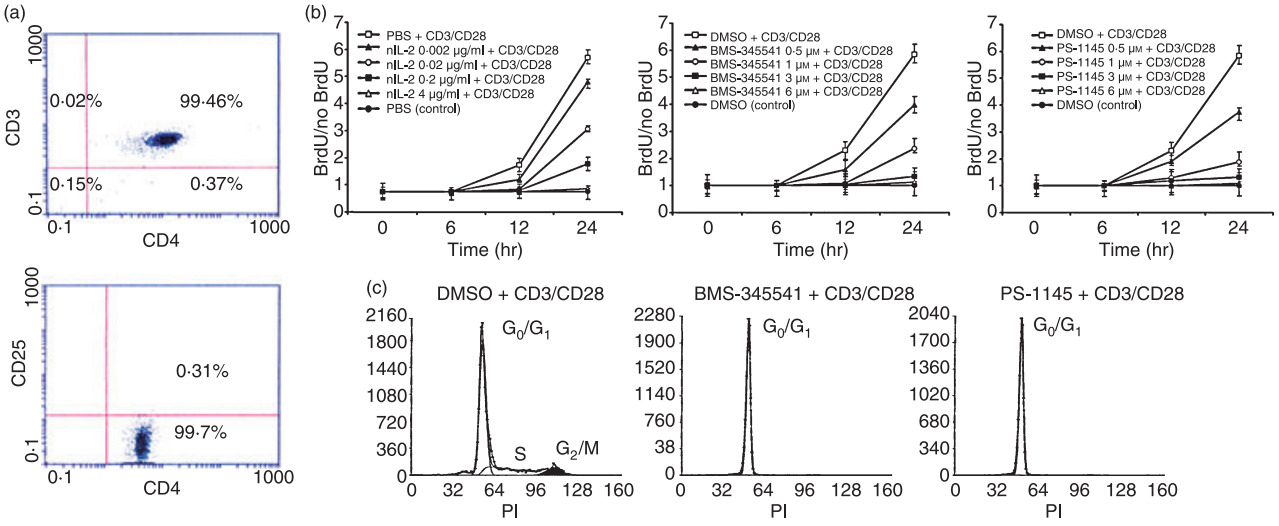

Figure 1. Human naïve CD4+ T-cell purification and proliferation. (a). Flow cytometric analysis of freshly isolated and CD25+ depleted CD4+ T cells. (b). Effects of neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2), BMS-345541 and PS-1145 on proliferation of CD28-costimulated T cells. Values are the mean ± standard deviation (SD) of three independent experiments, each carried out in duplicate. (c). Flow cytometric analysis of DNA content in control and in BMS-345541 and PS-1145 pretreated human naïve CD4+ T cells following 24 hr stimulation. PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide; PI, propidium iodide.

At 4 μg/ml, nIL-2 abrogated T-cell proliferation. nIL-2 effects were reproduced by the two IKK inhibitors, BMS-345541 and PS-1145, at increasing concentrations. At 3 μM, both inhibitors reduced cell proliferation by over 90%, at all times tested (Fig. 1b).

Analysis of DNA content showed that BMS-345541 and PS-1145 inhibited cell-cycle progression before DNA synthesis. Inhibition of cell proliferation was not caused by pro-apoptotic effects, as shown by the absence of hypodiploid DNA peaks left of the G0/G1 peak (Fig. 1c).

IKK inhibition hampers I-κBα degradation, NF-κB nuclear translocation and IL-2 and IL-2RA up-regulation

CD3/CD28 costimulation of human naïve CD4+ T cells was associated with a marked decrease in I-κBα levels. I-κBα was significantly stabilized in cells pretreated with BMS-345541 or PS-1145 (Fig. 2a). CD3/CD28 costimulation also resulted in noticeable nuclear translocation of both p50 and p65-RelA, which was markedly reduced by pretreatment with either drug (Fig. 2b-e).

To further evaluate the effects of IKK inhibition on T-cell activation, the expression of IL-2, a c-Rel-responsive gene, and IL-2RA, a RelA-p50-c-Rel-responsive gene,[18] was evaluated in costimulated T cells, with or without pretreatment with BMS-345541 and PS-1145. As shown in Fig. 3, CD3/CD28 costimulation was associated with the up-regulation of IL-2 and IL-2RA genes, which was markedly reduced by BMS-345541 and PS-1145. Taken together, these results demonstrate that, in the selected experimental settings, BMS-345541 and PS-1145 effectively inhibit the activation of the canonical NF-κB signalling pathway.

IKK inhibition negatively affects IL-2-independent expression of cell-cycle regulatory proteins

As BMS-345541 and PS-1145 inhibition of human naïve CD4+ T-cell proliferation was closely linked to reduced up-regulation of IL-2 and IL-2RA, one could speculate that the two inhibitors prevent T-cell expansion mainly by impairing IL-2-driven proliferation. To test this hypothesis, the effects of nIL-2 at 4 μg/ml on G1-, G1/S- and S-phase cyclin/CDK complex expression were compared with the effects of BMS-345541 or PS-1145 at 3 μM. BMS-345541 and PS-1145 reproduced all the effects of nIL-2, and prevented the up-regulation of cell-cycle regulatory proteins that were unaffected by IL-2 neutralization.

Specifically, CD3/CD28 costimulation of T cells caused the induction of cyclins D2 and D3, and their associated kinases CDK4 and CDK6, as early as 12 hr post-stimulation at both the mRNA and protein levels. nIL-2 suppressed, in a dose-dependent manner, cyclin D2 and CDK6 induction, and reduced CDK4 expression by approximately 50%, but did not alter the expression of cyclin D3. In contrast, BMS-345541 and PS-1145 abrogated the induction of both cyclins and both kinases (Figs 4a and 5a,c). Induction of cyclin E, cyclin A and CDK2 was detected after 24-hr of CD3/CD28 costimulation. nIL-2 prevented, in a dose-dependent manner, the induction of cyclin A, but did not affect the expression of cyclin E or CDK2. In contrast, the induction of cyclin A,

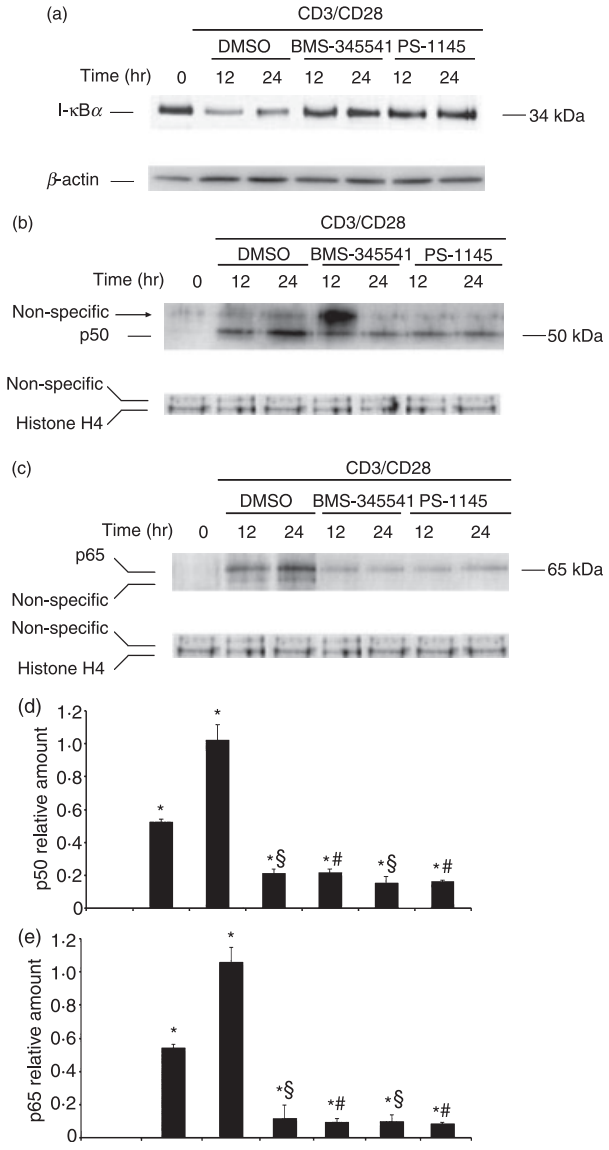

Figure 2. Effects of BMS-345541 and PS-1145 on degradation of I-κBα) and nuclear translocation of NF-κB p50 and p65-RelA in CD28-costimulated human naïve $CD4^+$ T cells. Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145 or drug vehicle [dimethylsulphoxide (DMSO)] and then stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. Equal amounts (10 μg/lane) of cytosolic (a) or nuclear (b and c) proteins were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with 0·4 μg/ml antibody against I-κBα (a), NF-κB p50 (b) and p65-RelA (c). Blots were stripped and re-probed with anti-β-actin (cytosolic extracts) or anti-histone H4 (nuclear extracts) to verify equal protein loading. One experiment is shown. Blots from three independent experiments were quantified, and the mean ± standard deviation (SD) are shown in the bar graphs for p50 (d) and p65 (e). The arrow indicates a non-specific band. Statistical significance: $^*P < 0{\cdot}05$ versus control cells. $^{\S}P < 0{\cdot}05$ versus DMSO-treated stimulated cells (12 hr). $^{\#}P < 0{\cdot}05$ versus DMSO-treated stimulated cells (24 hr).

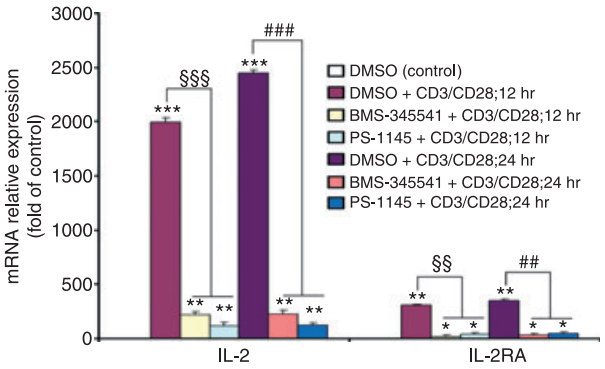

Figure 3. Effects of BMS-345541 and PS-1145 on interleukin (IL)-2 and interleukin-2 receptor, alpha chain (IL-2RA) gene expression in CD28-costimulated human naïve $CD4^+$ T cells. Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145 or drug vehicle [dimethylsulphoxide (DMSO)], and stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. IL-2 and IL-2RA mRNAs were quantified by real-time polymerase chain reaction (PCR). Expression is shown as the fold difference compared with the same gene in DMSO-treated cells (control). Values are the mean ± standard deviation (SD) of three independent experiments. Because of the large *y*-axis scale, control values (set to 1) are not appreciable. Statistical significance: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ versus control cells. $^{\S\S}P < 0{\cdot}01$; $^{\S\S\S}P < 0{\cdot}001$ versus DMSO-treated stimulated cells (12 hr). $^{\#\#}P < 0{\cdot}01$; $^{\#\#\#}P < 0{\cdot}001$ versus DMSO-treated stimulated cells (24 hr).

cyclin E and CDK2 was prevented by BMS-345541 and PS-1145 (Figs 4b and 5b,c). These data suggest that, in naïve $CD4^+$ T cells activated through 24-hr engagement of the TCR and the CD28 co-receptor, the CD28/IKK signalling pathway controls the expression of cyclin D3, cyclin E and CDK2, whereas the IL-2 signalling pathway regulates the expression of cyclin D2, cyclin A and CDK6. The expression of CDK4 is under the combined control of both pathways (Table 1).

Addition of exogenous recombinant human interleukin-2 up to 50 U/ml could not overcome the inhibitory effects of BMS-345541 or PS-1145 (not shown).

IKK inhibition prevents $p27^{KIP1}$ degradation by affecting SKP2 and CKS1B stability

CD3/CD28 costimulation of human naïve $CD4^+$ T cells resulted in a drastic reduction in $p27^{KIP1}$ as early as 12 hr post-stimulation which was prevented by nIL-2, BMS-345541 or PS-1145 (Fig. 6).

The F-box protein SKP2 and its co-factor CKS1B, the two proteins responsible for the recruitment of phosphorylated $p27^{KIP1}$ to the $SCF^{SKP2\text{-}CKS1B}$ ubiquitin ligase complex, are absent in G0, but their levels rise during G1 and S phase, because of increased gene expression and protein stability.[33,34] In resting T cells, SKP2 and CKS1B were undetectable, but their expression was induced after 12

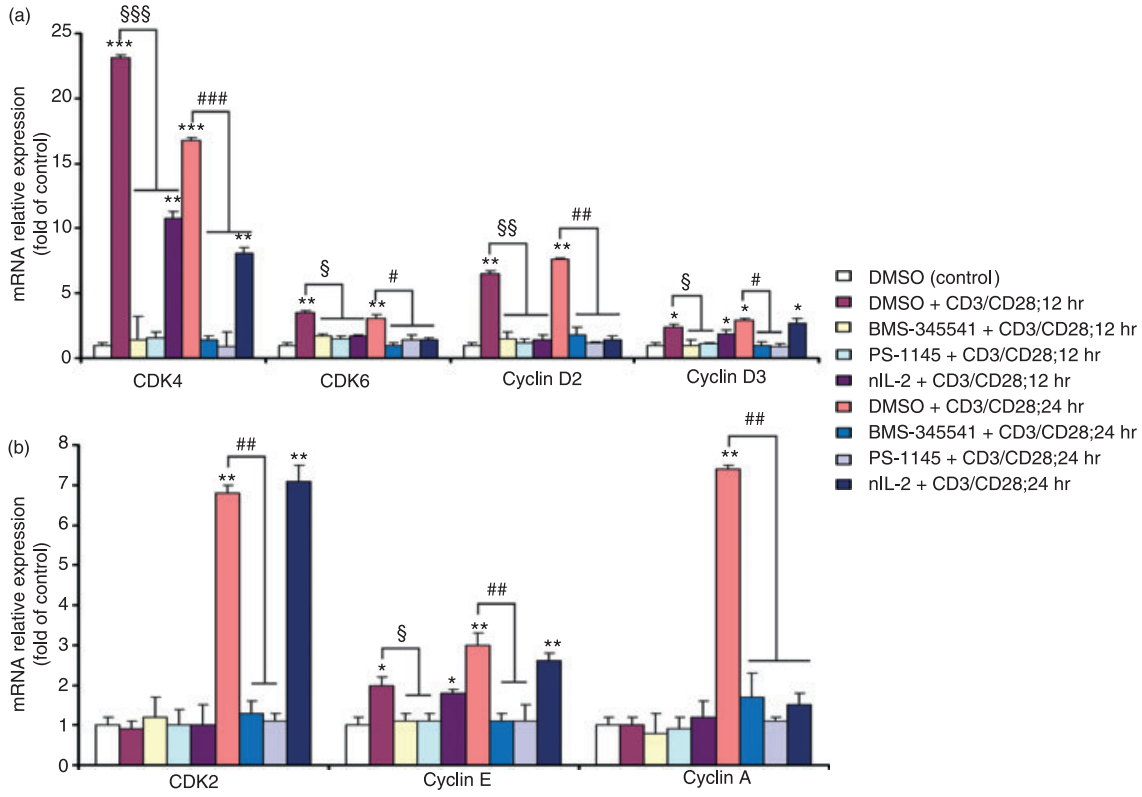

Figure 4. I-κB kinase (IKK) inhibition and interleukin (IL)-2 neutralization impair the induction of cell-cycle regulatory genes required for G0/G1 phase transition (a) and S phase entry (b). Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145, or 4 μg/ml neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2) or drug vehicle [dimethylsulphoxide (DMSO)], and stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. mRNAs were quantified by real-time polymerase chain reaction (PCR). Expression is shown as fold difference compared with the same gene in DMSO-treated cells (control). Values are the mean ± standard deviation (SD) of three independent experiments. Statistical significance: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ versus control cells. $^{\S}P < 0{\cdot}05$; $^{\S\S}P < 0{\cdot}01$; $^{\S\S\S}P < 0{\cdot}001$ versus DMSO-treated stimulated cells (12 hr). $^{\#}P < 0{\cdot}05$; $^{\#\#}P < 0{\cdot}01$; $^{\#\#\#}P < 0{\cdot}001$ versus DMSO-treated stimulated cells (24 hr). CDK, cyclin-dependent kinase.

and 24 hr of costimulation. nIL-2 did not affect induction of SKP2 and CKS1B, either at the mRNA or protein level. In contrast, in the presence of BMS-345541 or PS-1145, the two proteins were undetectable, although the up-regulation of their coding mRNAs was preserved (Fig. 7a–d).

BMS-345541 and PS-1145 pretreatment is not associated with a general block of protein expression

The effects of BMS-345541 and PS-1145 pretreatment on the expression of lamin-B1, β-actin, GAPDH, proteasome subunit alpha type 5 and β-tubulin, which are up-regulated in CD3/CD28-costimulated T cells,[35,36] were examined. As shown in Fig. 8a, upon CD3/CD28 costimulation, the expression of all proteins was equally up-regulated in T cells, regardless of BMS-345541 or PS-1145 pre-treatment.

The expression of the EGR-2 transcriptional regulator is rapidly induced in CD3- and in CD3/CD28-costimulated T cells through the nuclear factor of activated T cell (NFAT) signalling pathway.[37] In CD3/CD28-costimulated human naïve T cells, EGR-2 expression peaked at 12 hr post-stimulation, and rapidly decreased in the following 12 hr. Similar kinetics were seen in BMS-345541 and PS-1145 pretreated cells (Fig. 8b).

Discussion

Proliferation of naïve T cells in response to a short (20–24 hr) engagement of the TCR and the CD28 co-receptor critically relies on the up-regulation of IL-2 and of its high-affinity receptor IL-2RA.[3,23,24]

In this study we used a neutralizing anti-human IL-2 antibody and two selective, structurally unrelated, cell-permeable IKK inhibitors, BMS-345541 and PS-1145, to show that in human naïve $CD4^+$ T cells, in response to a short engagement of the TCR and the CD28 co-receptor, signals from IKK promote the up-regulation of IL-2 and IL-2RA genes, and control the expression of a set of cell cycle-regulatory proteins through mechanisms that are not mediated by IL-2. In these cells, exposed or not to BMS-345541 or

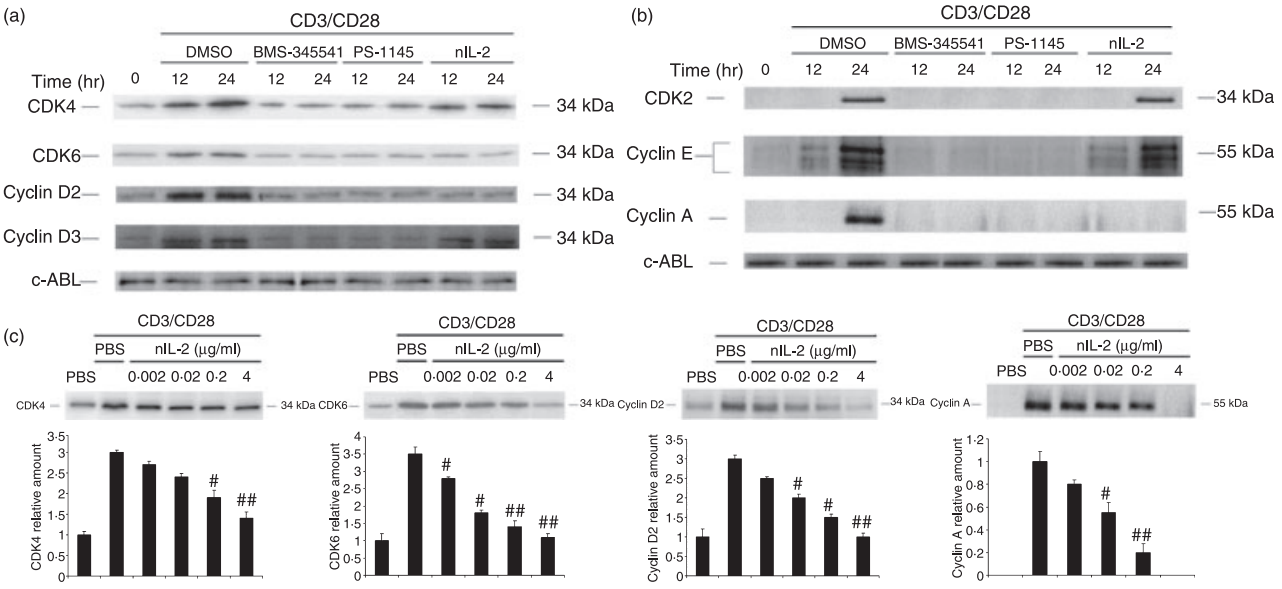

Figure 5. I-κB kinase (IKK) inhibition and interleukin (IL)-2 neutralization impair the expression of cell-cycle regulatory proteins required for G0/G1 phase transition (a) and S phase entry (b). Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145, or 4 μg/ml neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2) or drug vehicle [dimethylsulphoxide (DMSO)], and then stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. Equal amounts of proteins (20 μg/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using Tris/Glycine [cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, cyclin A and cyclin E] or Tris/Tricine (cyclin D2 and cyclin D3) buffer systems and immunoblotted with the specified antibodies at 0·4 μg/ml. Blots were stripped and re-probed with an antibody against c-ABL (c-abl oncogene 1) to verify equal protein loading. The results are representative of three independent experiments. Titration of nIL-2 (c). Human naïve $CD4^+$ T cells (3×10^6) were added with 0·002–4 μg/ml nIL-2 or drug vehicle (PBS) and then stimulated for 24 hr with anti-CD3 plus anti-CD28 antibodies (CD3/CD28). Equal amounts of proteins (20 μg/lane) were subjected to immunoblotting with the specified antibodies at 0·4 μg/ml as described in (a) and (b). One experiment is shown. Blots from three independent experiments were quantified, and the mean ± standard deviation (SD) are shown in the bar graphs. Statistical significance: $^{\#}P < 0{\cdot}05$; $^{\#\#}P < 0{\cdot}01$; versus stimulated cells. PBS, phosphate-buffered saline.

Table 1. Summary of the regulatory effects of CD28/ I-κB kinase (IKK) and interleukin (IL)-2 pathways on the expression of cell-cycle regulatory proteins

	CD28/IKK	IL-2
CDK4	+	+
CDK6	−	+
Cyclin D2	−	+
Cyclin D3	+	−
CDK2	+	−
Cyclin E	+	−
Cyclin A	−	+

+, positive regulator; −, no effect.
CDK, cyclin-dependent kinase.

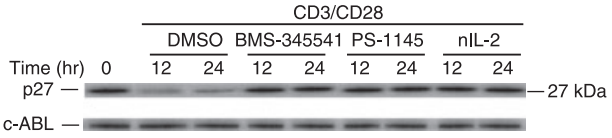

Figure 6. I-κB kinase (IKK) inhibition and interleukin (IL)-2 neutralization prevent $p27^{KIP1}$ down-regulation. Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145 or 4 μg/ml neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2) or drug vehicle [dimethylsulphoxide (DMSO)], and then stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. Equal amounts of proteins (20 μg/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with a $p27^{KIP1}$ specific antibody at 0·4 μg/ml. Blots were stripped and re-probed with an antibody against c-ABL (c-abl oncogene 1) to verify equal protein loading. The results are representative of three independent experiments.

PS-1145, the expression of proteins known to be up-regulated in activated T cells was comparable. Therefore, a general block of protein expression caused by BMS-345541 or PS-1145 toxicity can be excluded.

In activated T cells, cyclin D2 and cyclin D3 expression is rapidly and sequentially induced during G1 phase.[38] We also found that stimulation of human naïve $CD4^+$ T cells induced the expression of cyclin D2 and cyclin D3 at both the mRNA and protein levels. At saturating concentrations, nIL-2 abolished the induction of cyclin D2 but did not affect that of cyclin D3. Either BMS-345541 or PS-1145 prevented induction of both cyclins. These data are consistent with the reported role of IL-2 in up-regulation of the cyclin D2 gene,[39] and suggest that in activated

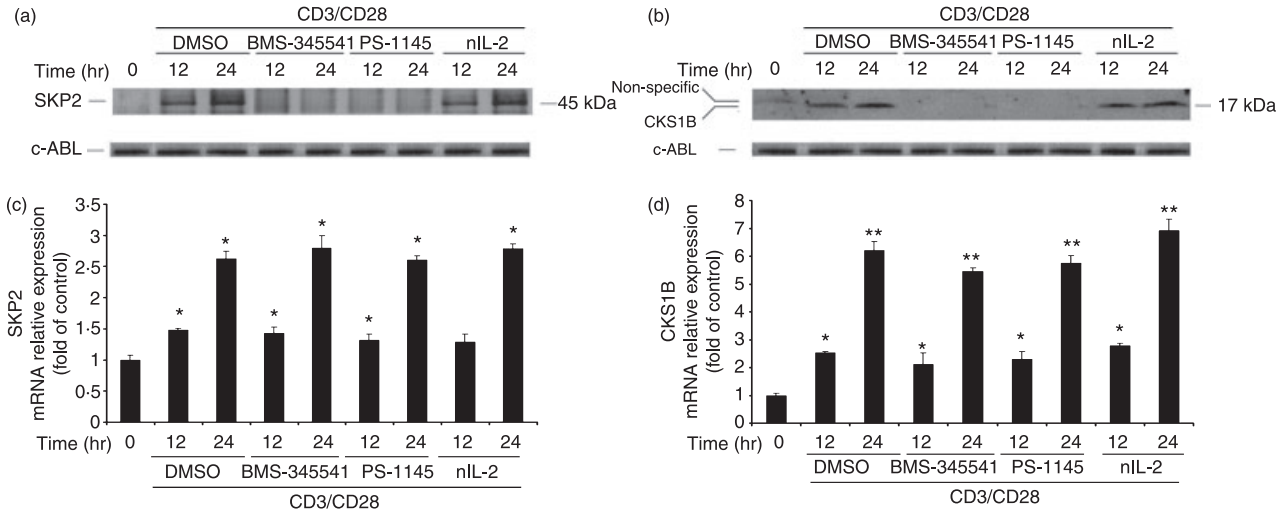

Figure 7. I-κB kinase (IKK) inhibition but not interleukin (IL)-2 neutralization negatively affects S-phase kinase-associated protein 2 (SKP2) (a) and CDC28 protein kinase regulatory subunit 1B (CKS1B) (b) stability. Human naïve CD4+ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145 or 4 μg/ml neutralizing anti-human interleukin-2 monoclonal antibody (nIL-2) or drug vehicle [dimethylsulphoxide (DMSO)], and then stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. Equal amounts of proteins (20 μg/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris/Tricine buffer system, and immunoblotted with the specified antibodies (1 μg/ml blocking solution). Blots were stripped and re-probed with an antibody against c-ABL (c-abl oncogene 1) to verify equal protein loading. The results are representative of three independent experiments. SKP2 (c) and CKS1B (d) mRNAs were quantified by real-time polymerase chain reaction (PCR). Expression is shown as fold difference compared with the same gene in DMSO-treated cells (control). Values are the mean ± standard deviation (SD) of three independent experiments. Statistical significance: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ versus control.

human naïve T cells most effects of IKK activation on cyclin D2 gene expression are mediated through the IL-2/IL-2R signalling pathway. These results also establish a direct, previously unknown link between IKK activation and the up-regulation of the cyclin D3 gene in human CD4+ T cells. In support of this, NF-κB binding sites in the cyclin D3 promoter of mouse CD4+ T cells have been reported.[40]

CDK4 and CDK6 were both induced upon CD3/CD28 costimulation. nIL-2 abrogated the up-regulation of CDK6, and partly inhibited CDK4 induction, while BMS-345541 and PS-1145 suppressed the induction of both kinases. Taken together, these results emphasize that an important effect of IKK activation on CDK4 and CDK6 expression relies on IL-2/IL-2R signalling. However, as full CDK4 up-regulation requires the activation of IKK and IL-2 signalling, these data add new information about the mechanisms that govern CDK4 expression in human T cells.

CDK2–cyclin E/A complexes are implicated in the regulation of major processes governing the G1/S transition.[5] In our experiments, CDK2 induction was detected in 24-hr costimulated cells, and was preserved in the presence of nIL-2, but abolished by BMS-345541 and PS-1145. We thus conclude that, in activated T cells, CDK2 induction is independent of IL-2 signalling, and relies instead on IKK activation, which is a novel finding.

To acquire catalytic activity, CDK2 must bind to cyclin E (G1/S phase transition) or cyclin A (S phase).[5] We found that T-cell stimulation caused a significant increase in cyclin E and cyclin A gene expression. nIL-2 prevented cyclin A up-regulation but did not affect cyclin E, a clear indication that in activated human naïve CD4+ T cells only cyclin A expression is dependent on the IL-2/IL-2R signalling pathway, consistent with previous reports.[3] Interestingly, BMS-345541 and PS-1145 prevented the expression not only of cyclin A, but also of cyclin E, providing compelling evidence for involvement of IKK in the regulation of cyclin E expression in human naïve CD4+ T cells. In light of the essential role played by the CDK2/cyclin E complex in initiating DNA replication,[5] this finding underscores a critical function of IKK in the regulation of T-cell entry into S phase.

Degradation of $p27^{KIP1}$ by the ubiquitin–proteasome pathway at the G0/G1 transition results in activation of the cyclin E/CDK2 complex, and commitment of cells to S phase.[41] In our results, stimulation of human naïve CD4+ T cells resulted in a considerable decrease in $p27^{KIP1}$ that was prevented by nIL-2, or BMS-345541 or PS-1145. The degradation of $p27^{KIP1}$ is a complex process that requires the formation of a ternary complex with cyclin D/CDK4, followed by $p27^{KIP1}$ phosphorylation on Thr187 by cyclin E/CDK2.[4] The RING finger-type ubiquitin ligase complex $SCF^{SKP2\text{-}CKS1B}$ recognizes phosphorylated $p27^{KIP1}$ through the C-terminus of two of its subunits, SKP2 and CKS1B, resulting in targeting of

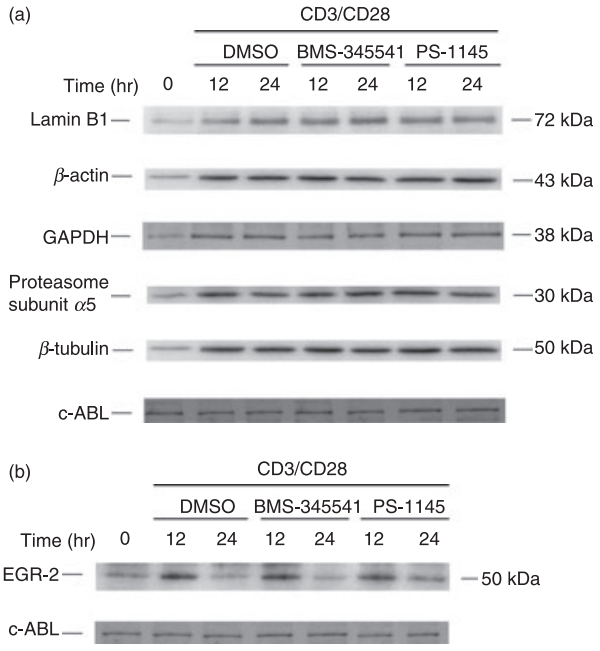

Figure 8. BMS-345541 and PS-1145 pretreatment is not associated with a general block of protein expression. (a, b) Human naïve $CD4^+$ T cells (3×10^6) were pretreated for 1 hr with 3 μM BMS-345541 or PS-1145 or drug vehicle [dimethylsulphoxide (DMSO)], and then stimulated with anti-CD3 plus anti-CD28 antibodies (CD3/CD28) for the indicated times. Equal amounts of proteins (20 μg/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with the indicated antibody (0·4 μg/ml). Blots were stripped and re-probed with an antibody against c-ABL (c-abl oncogene 1) to verify equal protein loading. The results are representative of three independent experiments. EGR-2, early growth response gene 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

$p27^{KIP1}$ for ubiquitination and degradation.[42] SKP2 and CKS1B levels periodically oscillate during the cell cycle: they are low or absent during G0 and early G1 phases, increase in late G1 phase, and peak in S phase, dropping as cells proceed through M and early G1 phases. Fluctuations in mRNA expression and degradation by the ubiquitin–proteasome pathway are responsible for variations in the levels of SKP2 and CKS1B.[33,34] We found that T-cell activation caused a 2·5-fold induction of SKP2 mRNA and a 6-fold induction of CKS1B, and the same occurred in cells exposed to nIL-2, BMS-345541 or PS-1145. Therefore, we conclude that, at the transcriptional level, SKP2 and CKS1B are not influenced by the functional status of IKK or IL-2 signalling. However, at the protein level, SKP2 and CKS1B expression was unaffected by nIL-2, but suppressed by BMS-345541 and PS-1145. Thus, we further conclude that, in stimulated human naïve $CD4^+$ T cells, IKK activation is crucial for the stability of the F-box protein SKP2 and its co-factor CKS1B. As phosphorylation of SKP2 on serine 64/72 is required for its stabilization and protection from anaphase-promoting complex (APC)^{Cdh1}-mediated degradation,[43,44] we propose that IKK activation assists, or is required for, this stabilizing mechanism in human T cells.

Inhibition by BMS-345541 or PS-1145 appears to be specific, because expression of β-actin, β-tubulin, lamin-B1, GAPDH and proteasome subunit α5 was similar in costimulated T cells with and without pretreatment, which excludes a general block in protein expression by either drug. This was supported by the comparable levels of induction seen for the NFAT-regulated EGR-2 transcription factor.

While PS-1145 is essentially an IKKβ inhibitor with a 50% inhibitory concentration (IC_{50}) of 0·15 μM, BMS-345541 can inhibit IKKβ and IKKα, although with different IC_{50s}: 0·3 μM for IKKβ and 4 μM for IKKα.[45] Therefore, the observations of the present study appear to result mainly from the inhibition of IKKβ, although the possibility of a contribution from IKKα inhibition cannot be formally excluded. BMS-345541 and PS-1145 are structurally unrelated, and share the unique, non-specific target, ERK-8 protein kinase.[46] As this is virtually absent in circulating leucocytes[47] our results are presumably not caused by the inhibition of kinases other than IKK.

Both the pharmacological inhibition of IKK[48] and the genetic repression of NF-κB proteins through the expression of a dominant negative form of I-κBα[49] are associated with markedly impaired proliferative responses of T cells, although the mechanisms by which this occurs are unclear. By demonstrating the ability of IKK-mediated signals to regulate transcription of cyclin D3, CDK2 and cyclin E, and protein stability of SKP2 and its co-factor CKS1B through IL-2-independent mechanisms, this study provides new information about the function of IKK in T-cell proliferation. However, with the exception of cyclin D3, no NF-κB binding sites have been reported in the promoters of the CDK2 or cyclin E genes. Therefore, no obvious explanation exists for the molecular mechanisms that link the pharmacological inhibition of IKK with the inhibition of CDK2 and cyclin E up-regulation in human T cells. Finally, in contrast to T cells expressing dominant negative forms of I-κBα,[49,50] human naïve $CD4^+$ T cells exposed to the pharmacological inhibitors of IKK did not result in an increased susceptibility to apoptosis consistent with previous reports on natural killer (NK) T cells.[48] This presumably reflects the different levels of residual NF-κB activity in each experimental system.

In conclusion, the present study exploited the potency and selectivity of two IKK inhibitors to show that IKK controls, in an IL-2-independent manner, the expression of several regulatory proteins crucial in enabling activated T cells to enter the cell cycle. Although further study is needed to thoroughly understand the mechanisms by which IKK regulates the expression of these proteins, our results provide new information about the molecular basis of the immunosuppressive and anti-inflammatory

effects of IKK inhibition. Thus, these findings may prove helpful for developing new and more selective pharmacologically active molecules.

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Disclosures

None.

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